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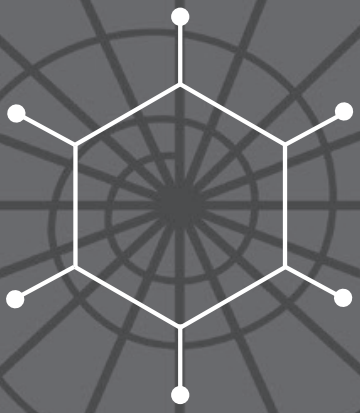
Construction and characterization of adenoviral vectors for the delivery of TALENs into human cells



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ABSTRACT

Transcription activator-like effector nucleases (TALENs) are designed to cut the genomic DNA at specific chromosomal positions. The resulting DNA double strand break activates cellular repair pathways that can be harnessed for targeted genome modifications. TALENs thus constitute a powerful tool to interrogate the function of DNA sequences within complex genomes. Moreover, their high DNA cleavage activity combined with a low cytotoxicity make them excellent candidates for applications in human gene therapy. Full exploitation of these large and repeat-bearing nucleases in human cell types will benefit largely from using the adenoviral vector (AdV) technology. The genetic stability and the episomal nature of AdV genomes in conjunction with the availability of a large number of AdV serotypes able to transduce various human cell types make it possible to achieve high-level and transient expression of TALENs in numerous target cells, regardless of their mitotic state. Here, we describe a set of protocols detailing the rescue, propagation and purification of TALEN-encoding AdVs. Moreover, we describe procedures for the characterization and quantification of recombinant viral DNA present in the resulting AdV preparations. The protocols are preceded by information about their underlying principles and applied in the context of second-generation capsid-modified AdVs expressing TALENs targeted to the *AAVS1* "safe harbor" locus on human chromosome 19.

INTRODUCTION

Targeted genome engineering is based on the creation of double-stranded DNA breaks (DSBs) at predefined chromosomal positions, which activate DNA repair pathways such as non-homologous end-joining (NHEJ) or the homologous recombination (HR) machinery (1). The potential sphere of action of genome engineering is broad and includes the development of new molecular medicine modalities based on the addition of therapeutic genes at so-called "safe harbor" loci or the correction of endogenous defective genes. Ultimately, such "genome surgery" should constitute a departure from conventional gene therapy, in which exogenous DNA is inserted at unpredictable chromosomal positions (2).

Generically, genome editing strategies depend on the introduction of designer nucleases into target cells for inducing the site-specific DSBs (3). Designer nuclease technologies are evolving at a rapid pace and include zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and, more recently, RNA-guided nucleases (RGNs) based on the prokaryotic CRISPR/Cas9 adaptive immune systems (4). While each of these platforms have their pros and cons, assessment of the specificities of ZFNs and RGNs revealed a considerable degree of off-target DNA cleavage (5-9). On the other hand, currently available data suggest that TALENs seem to be better tolerated in mammalian cells than ZFNs (10,11), although it is not clear yet whether "better tolerability" correlates with higher target site specificity, a key parameter in future therapeutic applications of genome editing protocols in patient-derived cells.

TALENs are easy to engineer heterodimeric designer nucleases that combine high cleavage activity with low cytotoxicity (12). The modularly structured DNA binding domain of transcription activator-like effectors (TALEs) was originally discovered in pathogenic bacteria of the genus *Xanthomonas* (13,14). Each repeat module within the DNA recognition domain consists of a conserved stretch of about 34 residues. The interaction with a single nucleotide of the DNA target site is mediated via two variable residues in positions 12 and 13. Repeat modules with different specificities can be fused into tailored arrays to direct the non-specific *Fok*I endonuclease domain to a predetermined target site (15,16).

Adenoviral vectors (AdVs) based on human adenoviruses (HAdVs) are widespread and versatile vehicles for introducing recombinant genes into human cells (17). Among these, those encoding immunomodulatory proteins or antigens are often selected as therapeutic payloads for anticancer and vaccination purposes (18). The broad use and adaptability of AdVs stem from several key features of their parental viruses as well as the extensive knowledge gathered on their biology since they were discovered in 1953 (19).

Presently, over 50 human adenovirus serotypes have been identified and classified in various sub-groups or species (A to G). They are mildly pathogenic viruses consisting of a linear protein-capped double-stranded DNA genome packaged in a non-enveloped

icosahedral capsid with fiber proteins protruding from each of its twelve vertices (17,18). The structural proteins are involved in host cell attachment by binding to specific cell surface receptors and can differ substantially among the various serotypes (20). As a result, the cell tropism of *Adenoviridae* family members can vary profoundly, owing in large part to their particular fiber-receptor interactions. For instance, under regular cell culture conditions, the prototypic species C serotypes 2 and 5 utilize the Coxsackievirus B and Adenovirus receptor (CAR) as primary cell surface attachment moiety (21), whereas CD46 is engaged by, amongst others, the species B serotypes 35 and 50 (22). An important feature of human adenoviruses is the similarity with which they infect diving and non-dividing cells. Indeed, the entry of viral genomes into host cell nuclei is independent of nuclear envelope integrity owing to the active process by which endosome-remodeled virions dock at the nuclear pore complex, disassemble and release their genomes into the nucleoplasm after having been transported in complex with dynein along the cytoplasmic microtubule network (23). Once in the nucleus, the orchestrated activation of the "early" (*E*) and "late" (*L*) viral gene expression programs yields large amounts of newly assembled mature particles. At the completion of the lytic viral life cycle, 10^3 to 10^4 virions are typically found within each host cell nucleus.

The most common AdVs are based on the CAR-interacting prototypic HAdV serotype 5 and are deleted in *E* regions whose products participate in viral DNA replication and/or activate the viral gene expression program (17). In addition to disrupting the regular replication cycle in target cells, these deletions create space for the insertion of exogenous DNA. As a result, for their production, these vectors require packaging cell lines, expressing *in trans* the viral genes, which have been deleted in the AdV backbone. These types of AdVs are called helper-independent as they can be amplified autonomously in such specialized packaging cell lines. The simplest helper-independent AdV type lacks the viral early region 1 (*E1*) and are thus called *E1*-deleted or first-generation AdVs. Notwithstanding the attributes of first-generation AdVs and their suitability for many different applications, it was shown that at high multiplicities of infection (MOI), the removal of *E1* does not fully block expression of viral open reading frames (ORFs), presumably due to partially complementing *E1A*-like cellular functions (24). Albeit at low-levels, the ensuing *de novo* viral protein synthesis can hamper the optimal deployment of these AdVs in certain experimental settings, such as those involving the transduction of therapeutically relevant cell types. This has prompted the development of second-generation AdVs combining deletions in *E1* with those in other *E* region(s), namely, early region 2A (*E2A*) and/or early region 4 (*E4*) (17). Of note, because the functions encoded in early region 3 (*E3*) are dispensable for vector propagation in cells cultured *in vitro*, first- and second-generation AdVs are often also deleted in *E3*. In addition to curtailing the phenomenon of "leaky" viral gene expression in target cells, propagation of second-generation AdVs is less likely to yield contaminating replication-competent AdVs (RCAs) resulting from recombination events between viral sequences shared by vector

and commonly used producer-cell genomes (25). Clearly, the issue of RCA contamination is particularly relevant whenever AdV preparations are produced for therapeutic gene transfer protocols (26). For this reason, AdV packaging cell lines, such as PER.C6 and N52.E6, were designed without DNA sequence overlap between the recombinant portions of their genomes and AdV DNA (27,28).

The available in-depth knowledge about adenovirus capsid structure and gene regulation allows constructing AdVs with genomic modifications not only in *E* but also in *L* ORFs. For instance, genetic manipulation of the fiber-encoding *L* transcription unit 5 (*L5*) permits retargeting AdV particles to cells with low or no CAR on their surface. These transductional targeting strategies can be based on engineering capsids with heterologous ligands (29) or on tapping the natural diversity of HAdVs to create chimeric fibers displaying receptor-interacting motifs derived from a serotype with a selected tropism of choice (29,30). This "fiber swapping" approach is based on exchanging the apical fiber motifs of serotype 5 for those of another serotype, such as serotype 35 or serotype 50, and has proven to be very valuable for the transduction of therapeutically relevant CAR-negative cells. These cells include normal and malignant hematopoietic cells as well as myocytes and mesenchymal stromal cells (20,29,30).

We postulate that AdV technologies, besides their utility in established biomedical research areas such as oncology and vaccinology, will start playing an increasing role in the emerging field of genome engineering as well. In fact, AdV-mediated introduction of ZFNs into $CD4^+$ T cells to knockout the HIV co-receptor gene *CCR5*, represents the first testing of a designer nuclease-based therapeutic strategy in humans (31). Related to this, our laboratories together with collaborators have recently demonstrated that AdVs can introduce functional *TALEN* ORFs into human cells (32). Importantly, the frequencies of targeted mutagenesis achieved by AdV-mediated TALEN expression were similar in transformed and non-transformed cells (32). In contrast, the same *TALEN* ORFs delivered in the context of conventional and integration-defective lentiviral vectors suffered from extensive function-impairing rearrangements within their repeat arrays, presumably due to frequent template switching of the reverse transcriptase (32). Yang and colleagues have subsequently confirmed these results by demonstrating that lentiviral vector-mediated delivery of intact *TALEN* ORFs requires substantial sequence optimization to reduce the length and frequency of repetitive sequences (33). These optimized TALENs were dubbed re-coded TALENs. However, because there are precedents for the importance in achieving high yields of sequence-specific nucleases in target cells for robust site-specific DSB formation (34,35), AdVs might ultimately be preferable over silencing-prone IDLVs (36) for transient TALEN expression.

In the current article, we provide a set of protocols detailing the production and purification of AdVs encoding TALENs. In addition, we describe in a comprehensive manner

procedures for the characterization and quantification of AdV genomes. These protocols are applied in the context of producing and validating a pair of second-generation fiber-modified AdVs containing *TALEN* ORFs under the control of the house-keeping *PGK1* gene promoter (PGK). The resulting TALEN proteins are addressed to target sequences within the so-called *AAVS1* "safe harbor" locus, located in the long arm of the human chromosome 19 at position 19q13.42-qter. Of note, the herein described methodologies can be directly applied to the generation and characterization of AdV particles harboring other transgenes of interest, including those encoding other types of sequence-tailored nucleases or genome-modifying enzymes.

CONSTRUCTION OF ADV MOLECULAR CLONES

Materials

1. Electro-competent *E. coli* strain BJ5183 (Addgene bacterial strain 16398) containing the pAdEasy-1 "backbone" plasmid or derivatives (here BJ5183$^{pAdEasy\text{-}2.50}$)
2. *E. coli* strain DH5α (Life Technologies)
3. pAdShuttle expression plasmids (e.g. pAdSh.PGK.SV40)
4. TALEN-encoding plasmids (e.g. 1383.pVAX.AAVS1.TALEN.L-94 and 1384.pVAX.AAVS1.TALEN.R-95)
5. 10 mg/ml transfer RNA (tRNA) carrier (Sigma-Aldrich)
6. Luria-Bertani broth (LB; Life Technologies) medium and agar plates
7. 500 µg/ml kanamycin sulfate (Sigma-Aldrich)
8. PmeI (New England BioLabs) or MssI (Thermo Scientific)
9. Absolute ethanol (J.T. Baker)
10. Table-top Eppendorf centrifuge
11. Electroporator (here Gene Pulser electroporator [Bio-Rad])
12. Agarose gel electrophoresis reagents and apparatus
13. Plasmid DNA purification system e.g. JETSTAR 2.0 Plasmid Maxiprep Kit (Genomed)
14. Spectrophotometer (here NanoDrop ND-1000 [Thermo Scientific])

Methods

The methods for constructing helper-independent AdV genomes are numerous. Among the first were those based on assembling full-length AdV DNA in producer human cells by HR between co-transfected "shuttle" and "backbone" constructs. Alternative methods based on AdV genome assembly in yeast or bacteria cells were subsequently developed due to the inefficiency and unpredictability of DNA recombination in human cells (17). Among these procedures, the AdEasy system is a particularly simple and robust one owing to its reduced

dependency on enzymatic manipulations and reproducibility in recovering plasmids containing whole AdV DNA (37). From the onset these reagents correspond to individual molecular clones that can be directly applied for rescuing and propagating AdV particles in producer cells. This feature obviates the need for time-consuming plaque assays to isolate, screen and select viral clones prior to large-scale AdV propagation and purification. Recently, the versatility of this method was increased by the development of an AdEasy-based system to produce first- and second-generation AdVs with tropism for CAR- or CD46-positive cells (38). In this chapter, we exploit this new AdV assembly system for detailing the construction and characterization of second-generation fiber-modified AdVs containing *TALEN* expression units. The same methods can be applied to generate AdVs with other genotypes.

1- Insert each *TALEN* ORF into an AdEasy-compatible pAdShuttle expression plasmid by using conventional recombinant DNA procedures (**Fig. 1**) (39). In the present work, the *TALEN* ORFs in 1383.pVAX.AAVS1.TALEN.L-94 and 1384.pVAX.AAVS1.TALEN.R-95 (32) were inserted into pAdSh.PGK.SV40 yielding expression plasmids pAdSh.PGK.TALEN-L^{S1} and pAdSh.PGK.TALEN-R^{S1}, respectively. Of note, the decision on which promoter to use is an important one and should be done having in sight which cell type(s) are intended as target cells. For instance, it is known that the ubiquitously used cytomegalovirus *immediate-early* gene promoter, albeit strong in many cell types, underperforms or suffers from silencing phenomena in others. Among the latter are those with substantial scientific and therapeutic relevance such as muscle cells and embryonic stems cells. Therefore, to broaden the use of transient AdV-mediated TALEN expression in human cells, it might be advisable to select cellular gene regulatory elements. Generally, valuable candidates are represented by the promoter/enhancer elements derived from the human genes *EEF1A1*, *UBC* and *PGK1* (a.k.a. *EF1*α, *ubiquin C* and *PGK*, respectively).

2- Digest 4 µg of TALEN-encoding pAdShuttle plasmids (e.g. pAdSh.PGK.TALEN-L^{S1} and pAdSh.PGK.TALEN-R^{S1}) with the restriction enzyme PmeI (or MssI isoschizomer) in a total volume of 40 µl. Check the completeness of the digestion by subjecting a 10-µl sample to agarose gel electrophoreses.

3- Precipitate the digested DNA by adding 20 µl of demineralized water, 25 µg of tRNA "carrier" molecules plus 2.5 volumes of ice-cold absolute ethanol, homogenize and centrifuge the mixtures at 20,000 ×g for 30 minutes at 4ºC.

4- Dissolve the recovered dried DNA pellet in 30 µl of demineralized water and use 10 µl to transform, in an ice-cold 1-mm cuvette, a 90-µl suspension of electro-competent BJ5183 cells containing the pAdEasy "backbone" of choice. In this chapter, to package the TALEN expression units in second-generation fiber-modified AdV particles, we used $BJ5183^{pAdEasy\text{-}2.50}$ cells (38). BioRad Gene Pulser electroporation settings: 1.25 V, 200 Ω and 25 µFD with a time constant of about 4 seconds.

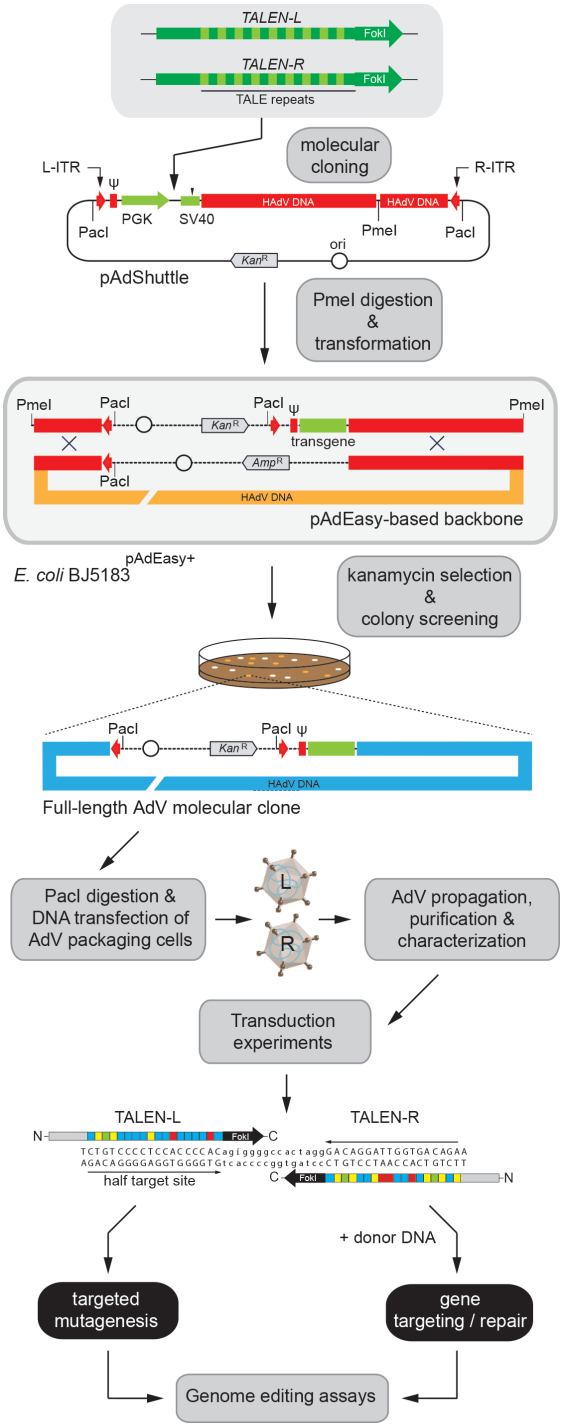


Figure 1. Outline of the AdV-based TALEN packaging and delivery system. The ORFs coding for a functional heterodimeric TALEN pair (*TALEN-L*/*TALEN-R*) are individually cloned into the multiple cloning site of an AdEasy "shuttle" plasmid (pAdShuttle) containing the transcriptional regulatory elements of choice (e.g. the human *PGK1* gene promoter [PGK] and the simian virus 40 polyadenylation signal [SV40]). L-ITR and R-ITR, HAdV "left" and "right" inverted terminal repeats, respectively; Ψ, HAdV packaging signal; TALE repeats, transcription activator-like effector region coding for the sequence-specific DNA binding domain; TALEN-L and TALEN-R, "left" and "right" TALE nuclease, respectively; FokI, DNA portion encoding the non-specific nuclease domain of the homodimer-forming type IIS restriction enzyme FokI; *Kan*^{R}, kanamycin-resistance gene; *ori*, prokaryotic origin of replication. The resulting constructs are treated with PmeI and are transformed into recombinogenic *E. coli* strain BJ5183$^{pAdEasy+}$ harboring a pAdEasy-derived "backbone" construct of choice. In the current work we deployed BJ5183$^{pAdEasy-2.50}$ cells to generate *E1*- and *E2A*-deleted (i.e. second-generation) AdVs displaying particle-retargeting fiber motifs from HAdV serotype 50. HR at DNA sequences common to "shuttle" and "backbone" constructs (labeled in the red) results in the assembly of full-length AdV molecular clones. These plasmids are isolated by screening individual colonies grown in agar plates containing kanamycin. Transfection of AdV packaging cell lines with PacI-digested AdV molecular clones leads to the assembly of AdV particles containing TALEN-encoding expression units. The rescued vector particles can subsequently be propagated to high-titers in producer cells and purified from cellular components. The resulting stocks of AdV particles are then subjected to assays to establishing the quantity and integrity of their packaged recombinant genomes. After this final characterization, AdV preparations are ready for genome editing experiments based on transducing and expressing the functional TALEN pair (TALEN-L/TALEN-R) in human cells.

5- Add 400 µl of LB broth medium without antibiotics, transfer the bacteria to a sterilized Eppendorf tube and shake for 45-60 minutes at 30ºC.

6- Plate the transformed cells onto two LB agar plates containing 50 µg/ml of kanamycin sulfate and, after an overnight incubation period at 30ºC, select and grow small-sized colonies for 16-20 hours at 30ºC in the presence of 50 µg/ml of kanamycin sulfate.

7- Isolate the plasmids by using a conventional alkaline lysis DNA extraction protocol and, on the basis of restriction enzyme analyses, select clones containing full-length AdV genomes for large-scale plasmid DNA purification. To this end, any commercially available DNA extraction procedure based on ion-exchange chromatography columns can be deployed. Optional extra step: transform into and purify from a non-recombinogenic *E. coli* strain (e.g. DH5α) the full-length AdV molecular clones.

8- Determine the concentration and purity (A_{260}/A_{280}) of the resulting large-scale DNA preparations by spectrophotometry and confirm their integrity by restriction enzyme fragment length analyses. If warranted, complement these data through transgene-directed DNA sequencing analyses.

RESCUE AND PROPAGATION OF ADVS

Materials

1. Full-length AdV molecular clones (here pAdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and pAdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50}).
2. PacI restriction enzyme (New England Biolabs)
3. 1 mg/ml 25-kDa linear polyethylenimine (PEI; Polysciences) in H_2O
4. 0.5 M NaCl (Merck)
5. 5 ml Polystyrene Round-Bottom tubes (BD Falcon)
6. *E1*- and *E2A*-complementing AdV packaging cell line (here PER.E2A cells)
7. Dulbecco's modified Eagle's medium (DMEM; Life Technologies)
8. Non-heat inactivated fetal bovine serum (FBS; Life Technologies)
9. 1M $MgCl_2$ (Sigma-Aldrich)
10. 50 ml blue screw cap conical bottom tubes (Greiner Bio-One)
11. 0.05% trypsin-ethylenediaminetetraacetic (EDTA) solution (Life Technologies)
12. Phosphate-buffered saline (PBS) pH=7.4
13. 6-well plates, 75- and 175-cm^2 cell culture flasks (all from Greiner Bio-One)
14. 0.45 µm pore-sized filters (Pall Life Sciences)
15. Liquid N_2 and water baths

Method

In brief, full-length AdV molecular clones (e.g. pAdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and pAdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50}) are first linearized with PacI (**Fig. 2A**). Next, the PacI-treated DNA is introduced into AdV packaging cells by a polycation-based DNA transfection protocol. The restriction enzyme treatment removes the prokaryotic DNA sequences and positions the ITR-embedded HAdV origins of replication close to DNA termini necessary for the initiation of HAdV-dependent replication. The rescued genome-containing AdV particles are subsequently amplified to high-titers by serial propagation on increasing numbers of producer cells.

1- Digest 6.25 µg of each full-length AdV molecular clone with PacI in a total volume of 30 µl, after which heat-inactivate the restriction enzyme by a 20-minute incubation period at 65ºC.

2- Seed PER.E2A cells (40) or any another *E1*- and *E2A*-complementing cell line in wells of 6-well plates. In the case of PER.E2A cells, seed 1.25×10^6 cells in DMEM containing 10% FBS and 10 mM $MgCl_2$. Incubate overnight at 39ºC in a 10% CO_2 atmosphere. The mutant *E2A* gene in PER.E2A cells confers a thermo-sensitive phenotype to the encoded DNA-binding protein (DBP). Therefore, these cells are normally cultured at 39ºC (non-permissive temperature) to avoid DBP's cytotoxicity, whereas at the time of vector production, they are shifted to 34ºC (permissive temperature) to allow proper folding of the protein. Clearly, to rescue first-generation AdVs with conventional or retargeting fibers, *E1*-complementing cell lines such as 293 (41), 911 (42), or PER.C6 (27) suffice.

3- Add the 30-µl PacI digestion mixture to 190 µl of 150 mM NaCl in a 5 ml polystyrene round-bottom tube. In parallel, dilute 18.8 µl of a 1 mg/ml PEI solution in 150 mM NaCl to a final volume of 220 µl.

4- Add the PEI to the DNA solution, vortex vigorously for approximately 10 seconds and incubate for 20 minutes at room temperature to let the DNA-polycation complexes form. Next, dispense dropwise the colloidal suspension into the wells containing the AdV packaging cells.

5- After overnight incubation at 39ºC in a 10% CO_2 atmosphere, remove the transfection mixtures, add regular culture medium and incubate the cells at the permissive temperature of 34ºC in a 10% CO_2 atmosphere. Monitor the monolayers for the appearance of viral plaques and, ultimately, complete cytopathic effect (CPE) as defined by over 90% detached producer cells. Of note, in case the transfected cells become over-confluent stalling the development of full CPE, sub-culture them into a 75-cm^2 flask taking care to beforehand remove the trypsin by pelleting and re-suspending the produced cells in regular fresh medium.

6- Release the rescued AdV particles from the producer cells by subjecting the collected cell suspension in medium to three cycles of freezing and thawing. To this end, liquid N_2 and 37ºC water baths can be used.

7- Remove the cellular debris by centrifugation at 1,000 ×*g* for 10 minutes in a swing-out rotor and add supernatant fractions to 75-cm^2 culture flasks containing newly seeded producer cells at about 70-80% confluence. Of note, for optimal AdV propagation, it is advisable adjusting the inocula fractions to result in complete CPE of the producer cells within 2 to 3 days post-infection. As an optional extra step, the recovered supernatants can be further clarified by filtration through 0.45 µm pore-sized filters. To generate a research-scale AdV preparation, a minimum of 1 and a maximum of 3 propagation rounds are normally sufficient to yield enough clarified AdV supernatant material ("master virus seed stock") to use in at least one large-scale infection round comprising 18-20 175-cm^2 culture flasks containing producer cells at 70-80% confluence (~2×10^7 cells/flask).

8- Collect the producer cells in sterile 50 ml blue screw cap conical bottom tubes and centrifuge them at 2,000 ×*g* for 10 minutes in a swing-out rotor. Discard the supernatants, resuspend the producer cells in 40 ml of PBS and divide the cell suspensions in two sterile 50 ml blue screw cap conical bottom tubes. Move forward to the concentration and purification of the AdV particles or store the material at -80ºC.

CONCENTRATION AND PURIFICATION OF ADV PARTICLES

Materials

1. Ultracentrifuge (here Beckman Coulter LE-80K)
2. SW28 rotor (Beckman Coulter) or similar
3. VTI65.1 rotor (Beckman Coulter) or similar
4. Open-top polyallomer centrifuge tubes, 25×89 mm (Beckman Coulter)
5. Quick-Seal® polyallomer centrifuge tubes, 16×76 mm (Beckman Coulter)
6. Tube Sealer 342420 (Beckman Coulter) or similar apparatus
7. Amicon Ultra-15 Centrifugal Filter Devices with 100,000 MWCO (Millipore) or similar device
8. 5% (w/v) sodium deoxycholate (DOC; Merck) in demineralized water
9. 1.24 g/ml, 1.33 g/ml and 1.4 g/ml cesium chloride (CsCl; Merck) solutions in Tris-HCl pH 7.5 with 5% glycerol
10. 10 mg/ml DNaseI (grade II; Roche Applied Science)
11. 20G needles (BD microlance 3) and 5-ml syringes (BD plastipak)
12. Storage buffer (20 mM Tris-HCl pH 8.0; 25 mM NaCl and 5% glycerol)

Methods

In brief, the AdV particles are first released from producer cells by a mild detergent treatment. Next, they are concentrated and purified by two consecutive ultracentrifugation steps entailing CsCl block and continuous isopycnic density gradients. Banded genome-containing AdV particles (**Fig. 2B**) are subsequently subjected to a de-salting buffer exchange protocol based on ultrafiltration.

1- Add to 20-ml suspensions of producer cells in PBS the detergent DOC at a final concentration of 0.5% and incubate the mixtures at 37ºC for 30 minutes. Gently invert the cell lysates every 5-10 minutes for a thorough homogenization of the resulting viscous solution.

2- Add $MgCl_2$ and DNaseI at final concentrations of 40 mM and 8.7 µg/ml, respectively. Subsequently, incubate the mixtures at 37ºC for 30 minutes while gently inverting the cell lysates every 5-10 minutes for a thorough homogenization.

3- Centrifuge the producer cell lysates for 10 minutes at 2000 ×*g*. Next, recover the supernatants and centrifuge them for 10 minutes at 3000 ×*g*. The resulting clarified cell lysates are subsequently subjected to ultracentrifugation through block and continuous CsCl density gradients as follows.

4- Add, in a gentle manner, 20-22 ml of clarified supernatant material on top of block gradients consisting of 5 ml and 10 ml CsCl tires with densities of 1.4 g/ml and 1.24 g/ml, respectively. Open-top polyallomer centrifuge tubes with a 38-ml capacity can be used for this purpose.

5- Place the loaded centrifuge tubes in a SW28 swing-out rotor (or similar) and centrifuge them at 80,000 ×*g* for 2 hours at 10ºC. Of note, for proper counterbalancing during ultracentrifugation, the weight differences between tubes placed at opposite rotor positions should not exceed 0.02 grams.

6- Recover the AdV particles concentrated at the border between the two CsCl densities by pricking the centrifuge tubes with a 20G needle coupled to 5-ml syringe.

7- Transfer the recovered AdV material into 13.5-ml polyallomer Quick-Seal® tubes and fill them up completely with 1.33 g/ml CsCl solution. Next, heat-seal the tubes by using a pre-heated Tube Sealer 342420 (or similar device). In alternative to heat-sealing, use Opti-Seal® polyallomer centrifuge tubes instead. Of note, for proper counterbalancing during ultracentrifugation, the weight differences between tubes placed at opposite rotor positions should not exceed 0.02 grams after sealing.

8- Place the loaded and sealed centrifuge tubes in a VTi 65.1 vertical-tube titanium rotor (or similar) and centrifuge them at 300,000 ×*g* for 16 hours at 10ºC to subject the AdV particles to a self-generating continuous buoyant density gradient. Of note, disable the break option of the ultracentrifuge prior to stopping these runs.

9- Use a needle to create a hole at the top of the sealed centrifuge tubes after which retrieve the banded AdV particles (**Fig. 2B**) by piercing the tubes with 20G needles coupled to 5-ml syringes.

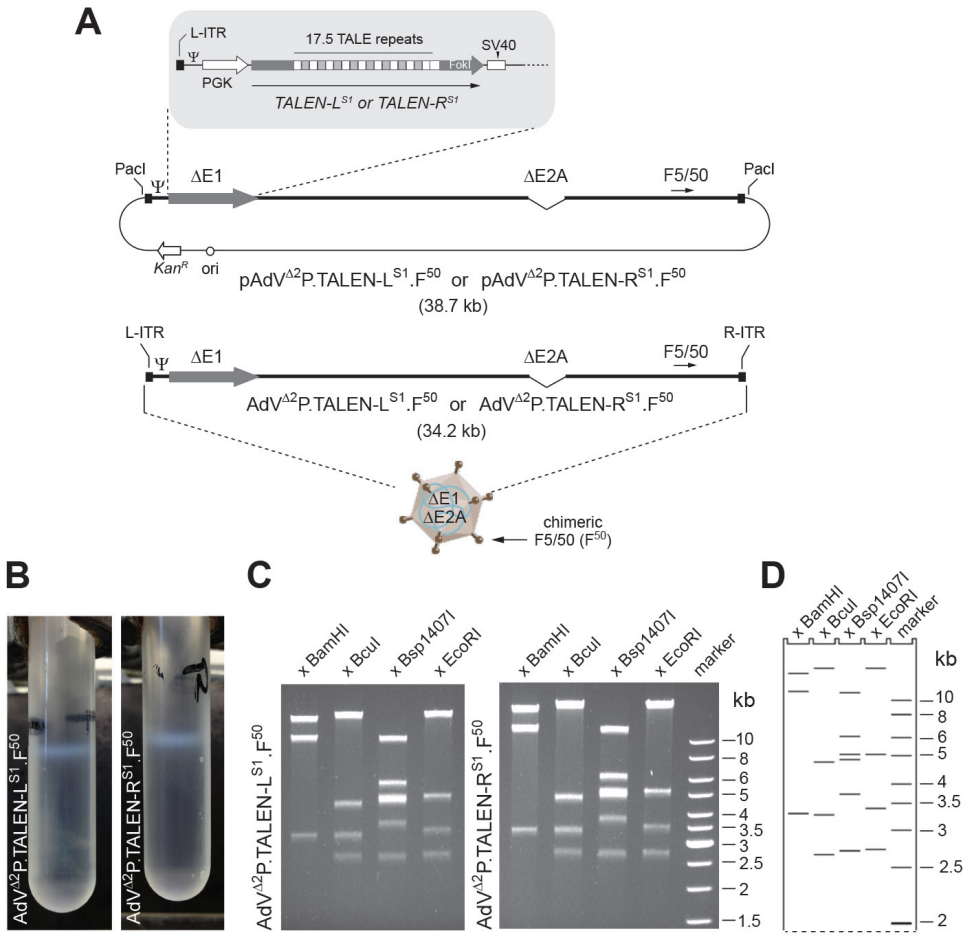


Figure 2. Analysis of recombinant genomes packaged in purified AdV particles. **(A)** DNA structures of second-generation (AdV$^{\Delta 2}$) fiber-modified (F^{50}) adenoviral vectors AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50} drawn in relation to those of their respective molecular clones pAdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and pAdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50}. The thick and thin lines correspond to the AdV and plasmid backbone sequences, respectively. F5/50, ORF coding for a chimeric fiber consisting of basal shaft domains from HAdV serotype 5 fused to apical shaft and knob motifs from HAdV serotype 50. For an explanation of the other elements see the legend of Fig. 1. These recombinants contain an expression unit coding for the *AAVS1*-specific designer nucleases TALEN-L^{S1} or TALEN-R^{S1}. The regulatory elements consist of the *PGK* promoter and the SV40 polyadenylation signal. The central portions of these *TALEN* ORFs encode an array of 17.5 repeats responsible for targeting the non-specific FokI nuclease domains to *AAVS1* to form an operative TALEN pair. (**B**) Aspect of concentrated genome-containing AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50} particles after isopycnic CsCl density-gradient ultracentrifugation. (**C**) Characterization of DNA isolated from CsCl-purified AdV particles by restriction fragment length analysis. Agarose gel electrophoresis of AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50} genomes (left and right panels, respectively) digested with BamHI, BclI, Bsp1407I or EcoRI. Marker, GeneRuler DNA Ladder Mix molecular weight marker (Fermentas). (**D**) Restriction patterns of AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50} DNA generated *in silico* with the aid of Gene Construction Kit (version 2.5) software (Textco BioSoftware, Inc.).

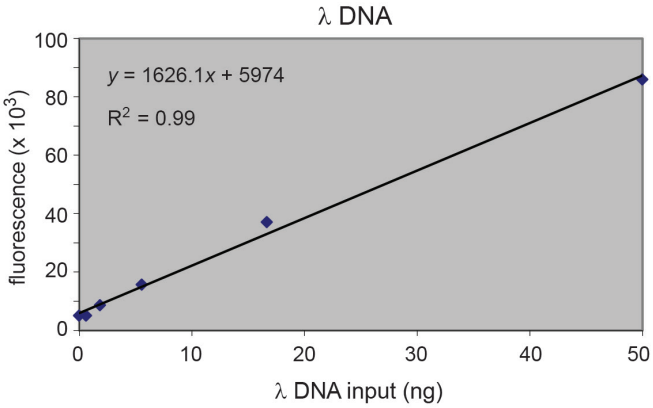
10- Transfer the recovered AdV material into Amicon Ultra-15 Centrifugal Filter Devices with a nominal molecular weight cut-off of 100 kDa previously rinsed with storage buffer.

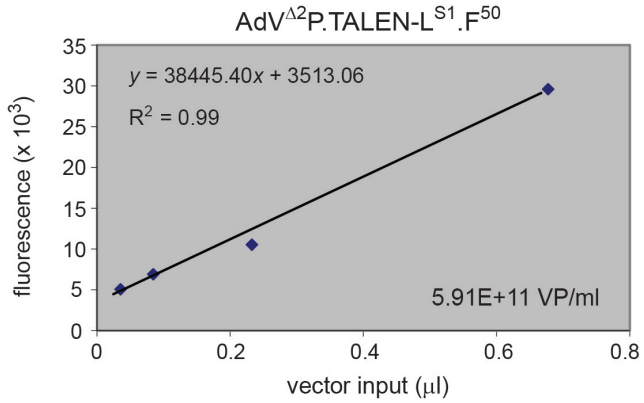
11- Fill-up the filter devices with storage buffer to their maximum 15 ml capacity and exchange the CsCl solution for the physiological storage buffer by carrying out a minimum of five consecutive cycles of centrifugation and addition of large volumes of fresh storage buffer. Of note, avoid drying the filter membrane by overextending the centrifugation step(s). As a guideline for the first centrifugation round, apply a centrifugal force of 2,000 ×*g* for 45 seconds at room temperature. For subsequent rounds adapt the centrifugation time depending on the retentate and flow-through volumes corresponding to each AdV preparation.

12- After the last cycle, recover the retentate (1.5-2.0 ml) into sterilized Eppendorf tubes or 5 ml polystyrene round-bottom tubes and rinse well the filter membranes 2-3 times with 200 µl of storage buffer. Pool this material into the bulk material, mix and make aliquots. Store the aliquots at -80ºC until their use.

CHARACTERIZATION OF ADV PREPARATIONS

The characterization of purified stocks of TALEN-encoding AdVs includes determining the integrity and amounts of packaged recombinant genomes (sections **5.1** and **5.2**, respectively) as well as validating the functionality of their expression units and encoded products (section **5.3**). After being extracted from AdV particles, the integrity of the vector genomes can be routinely examined by conventional restriction enzyme fragment length analyses (**Fig. 2C** and **Fig. 2D**). If necessary, these data can be complemented by more detailed analyses based on DNA sequencing of selected AdV DNA portions (e.g. *TALEN* ORFs). In addition, recombinant AdV genomes in purified preparations can be quantified by qPCR or, alternatively, by a quicker procedure based on disrupting AdV capsids with an ionic detergent and directly measuring the released vector DNA content with the aid of a dsDNA-specific fluorescent dye. Here we deploy such a method, adapted from Murakami and McCaman (43), to determine $AdV^{\Delta 2}$P.TALEN-L^{51}.F^{50} and $AdV^{\Delta 2}$P.TALEN-R^{51}.F^{50} titers in terms of genome-containing viral particles per ml (VP/ml). This method allows for accurate and rapid titer measurements by exploiting the sensitivity and wide dynamic range of the dsDNA-specific dye PicoGreen (**Fig. 3**). Finally, the functionality of AdV-delivered *TALEN* transcriptional units can be established by exploiting heterologous protein tags and standard western blot techniques (**Fig. 4A**) as well as surrogate NHEJ detecting assays (**Fig. 4B**) based on mismatch-sensitive endonucleases (e.g. T7 endonuclease I or CEL-1).





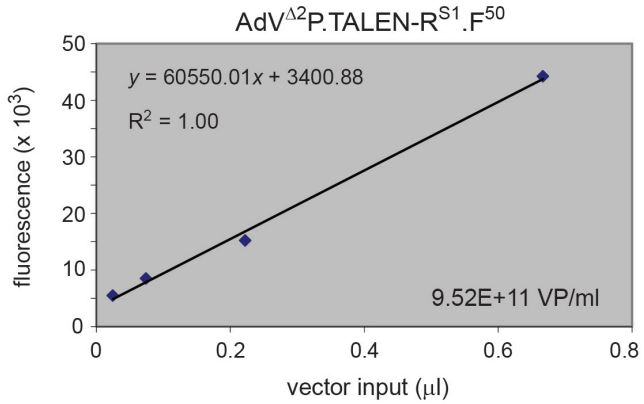


Figure 3. Fluorometric assay based on the double-stranded DNA dye PicoGreen for measuring AdV titers in terms of genome-containing viral particles per milliliter (VP/ml). Representative linear regression curves corresponding to a λ DNA standard curve and to DNA from CsCl-purified AdV stocks (i.e. AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50}) are depicted. The resulting AdV physical particle titers for each stock are also shown.

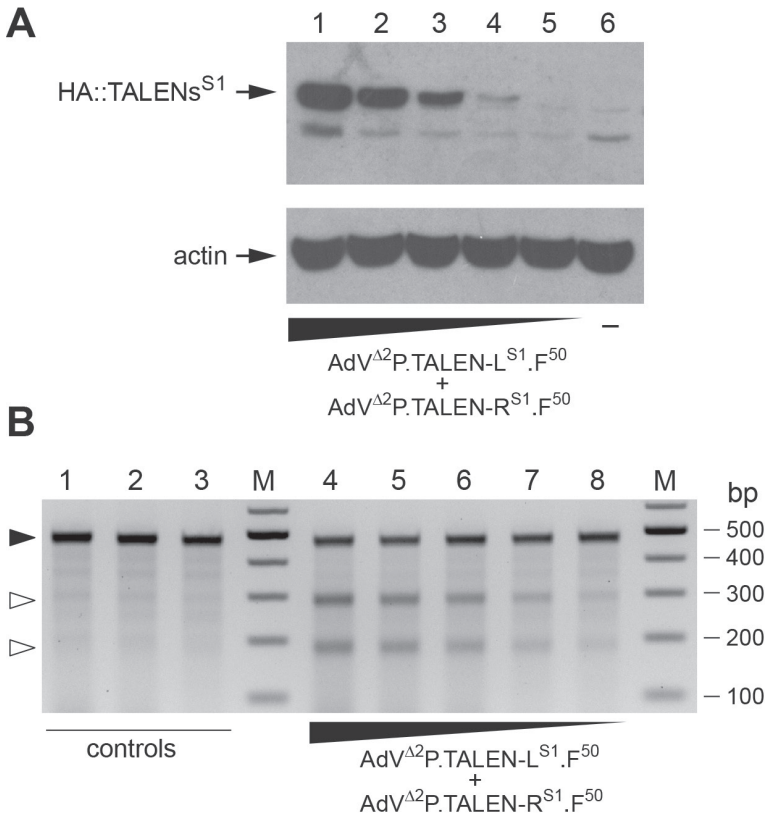


Figure 4. Transgene expression and targeted mutagenesis assays for the validation of TALEN-encoding AdV preparations. (**A**) Influenza hemagglutinin tag-specific Western blot analyses on protein lysates of human myoblasts co-transduced with 1:1 mixtures of AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{5} at total MOIs of 15×10^3, 10×10^3, 5×10^3, 2.5×10^3 and 1×10^3 VP/cell (lanes 1 through 5, respectively) or mock-transduced (lane 6). A parallel Western blot deploying an anti-actin mouse monoclonal antibody (clone: C4) provided for a loading control. (**B**) T7 endonuclease I genotyping assay to detect site-specific DSBs in target cells exposed for three days to TALEN-encoding AdVs. Human myoblasts were co-transduced with 1:1 mixtures of AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{5} at total MOIs of 15×10^3, 10×10^3, 5×10^3, 2.5×10^3 and 1×10^3 VP/cell (lanes 4 through 8, respectively). Controls were provided by mock-transduced human myoblasts (lane 3) or by human myoblasts exposed exclusively to 7.5×10^3 VP/cell of AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} (lane 1) or to 7.5×10^3 VP/cell of AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50,} (lane 2). Lanes M, GeneRuler DNA Ladder Mix molecular weight marker (Fermentas). PCR products spanning the *AAVS1* target site of control human myoblasts or of human myoblasts transduced with different doses of AdV$^{\Delta 2}$P.TALEN-L^{S1}.F^{50} and AdV$^{\Delta 2}$P.TALEN-R^{S1}.F^{50} are denatured and re-annealed. Heteroduplexes with indels resulting from NHEJ repair of DSBs at *AAVS1* bear mismatched base pairs that can be recognized and cleaved by T7 endonuclease I yielding low-molecular-weight DNA fragments whose positions are indicated by open arrowheads.

Structural analysis of packaged AdV genomes

Materials

1. 10× DNaseI buffer (130 mM Tris-HCl pH 7.5; 1.2 mM $CaCl_2$ and 50 mM $MgCl_2$)
2. 10 mg/ml DNaseI (Roche)
3. 0.5 mM EDTA pH 8
4. 20 mg/ml Proteinase K (Thermo Scientific)
5. 10% (w/v) SDS
6. Jetsorb Gel Extraction Kit (Genomed) or similar system
7. Selected restriction enzymes
8. Agarose gel electrophoresis reagents and apparatus

Methods

1- Add to a 50-µl aliquot of a CsCl-purified AdV preparation, 12 µl of 10× DNaseI buffer, 8 µl of 10 mg/ml DNaseI and 50 µl of demineralized water. Incubate the mixture for 30 minutes at 37ºC.

2- Inactivate the DNaseI by adding 2.4 µl of 0.5 mM EDTA pH 8, 6.0 µl of 10% SDS and 1.5 µl of 20 mg/ml proteinase K. Incubate the mixture for 1 hour at 55ºC.

3- Add 390 µl of buffer A1 (Jetsorb Gel Extraction Kit) and 10 µl of silica beads (Jetsorb Gel Extraction Kit). Incubate for 20 minutes and homogenize every 3-5 minutes by inverting the samples several times.

4- Centrifuge for 30 seconds at 20,000 ×*g*, discard the supernatant and gently resuspend the bead pellets in 400 µl of buffer A1.

5- Centrifuge for 30 seconds at 20,000 ×*g*, discard the supernatant and gently resuspend the bead pellets in 400 µf of buffer A2 (Jetsorb Gel Extraction Kit). Repeat this washing step and remove the supernatants as thoroughly as possible.

6- Air-dry the bead pellets until they become white. Of note, avoid over-drying the pellets to prevent difficulties in eluting the bound DNA.

7- Elute the DNA by adding 30 µl of nuclease-free demineralized water and incubate for 7 minutes at 55ºC. Increase the DNA elution by tapping the tubes approximately every 2 minutes.

8- Centrifuge the samples at 20,000 ×*g* for 30 seconds and carefully collect the supernatants containing the eluted AdV genomes. Of note, avoid carrying over silica beads as these can inhibit subsequent enzymatic reactions.

9- Perform restriction enzyme fragment length analysis to establish the integrity of the packaged AdV genomes (**Fig. 2C** and **Fig. 2D**) and, if warranted, carry out transgene-directed DNA sequencing.

Determination of AdV physical particle titers

Materials

1. Storage buffer (20 mM Tris-HCl pH 8.0; 25 mM NaCl and 5% glycerol)
2. Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies)
3. White 96-well microplate OptiPlate-96 (Perkin Elmer)
4. TE buffer (10mM Tris-HCl and 1 mM EDTA pH 7.5)
5. Spectrofluorometer plate reader (here Wallace 1420 VICTOR 3 multilabel plate reader, Perkin Elmer)

Methods

1- Dilute 5 µl of each AdV stock in 45 µl of storage buffer supplemented with 0.05% SDS. Next, prepare four 3-fold serial dilutions by mixing 15 µl of the vector material with 30 µl of storage buffer containing 0.05% SDS.

2- Incubate the dilution series for 5 minutes at 65ºC, after which pipet 20 µl of each of these samples into wells of white 96-well microplates.

3- Prepare a working solution of the fluorescent dye by diluting the stock of Quant-iT PicoGreen dsDNA 360 fold in TE buffer. Next, add 180 µl of this working solution to each of the dilutions. Incubate for 5 minutes in the dark at room temperature.

4- In parallel, set-up a calibration curve by serially diluting a stock of bacteriophage λ DNA (100 µg/ml) in storage buffer containing 0.05 % SDS to the final concentrations: 50, 16.7, 5.6, 1.9 and 0.6 ng/µl.

5- Measure the various DNA concentrations by deploying a Wallace 1420 VICTOR 3 multilabel plate reader (or similar device) set to excitation and emission wavelengths of 485 nm and 535 nm, respectively.

6- Generate linear regression curves for each of the measured data sets confirming that the corresponding coefficients of determination (R^2) lie between 0.98 and 1.0 (**Fig. 3**). Next, through the λ DNA standard curve correlating fluorescence signals to ng of DNA, determine for each AdV stock the amount of DNA (in ng) per µl of sample.

7- Calculate the molecular mass corresponding to each AdV DNA molecule by applying the relationship: Genome size (bp) × 660 (Da/bp) = Da ($gmol^{-1}$).

e.g. $AdV^{\Delta 2}P.TALEN\text{-}L^{S1}.F^{50}$ DNA = 34,155 bp × 660 Da/bp = 2.25×10^{7} $gmol^{-1}$.

8- Divide the resulting values by the Avogadro's number (i.e. 6.022×10^{23}) to obtain the weight of each AdV DNA molecule in grams and subsequently convert these values to ng; e.g. 2.25×10^{7} $gmol^{-1}$/6.022×10^{23} mol = 3.74×10^{-17} g → 3.74×10^{-8} ng

9- Determine the number of AdV genomes per ng; e.g. 1/3.74×10^{-8} ng = 2.67×10^{7} genomes

10- Use the values obtained in step **6** to calculate the total number of genomes per µl; e.g. 2.67×10^{7} genomes/ng × 22.1 ng/µl = 5.91×10^{8} genomes/µl → 5.91×10^{11} genomes/ml or genome-containing viral particles per ml i.e. VP/ml.

Functional validation of AdV-delivered TALENs

Materials

1. Cell lysis solution for genomic DNA extraction (100 mM Tris-HCl pH 8.5; 5 mM EDTA; 0.2% SDS and 200 mM NaCl)
2. TE-saturated phenol:chloroform:isoamylalcohol (25:24:1) solution (J.T. Baker)
3. Chloroform (Merck)
4. 7.5 M ammonium acetate (Merck)
5. Absolute ethanol (J.T. Baker)
6. 10 mM TE buffer pH 8.0
7. 10 mg/ml RNase A (Thermo Scientific)
8. 5U/µl GoTaq DNA polymerase (Promega)
9. 5× Colorless GoTaq Flexi buffer (Promega)
10. 40 mM deoxynucleotide (dNTP) solution mix (New England Biolabs) and 10 µM selected primer pair (Eurofin)
11. PCR thermocycler (here DNA Engine Tetrad 2 Peltier Thermal Cycler [Bio-Rad])
12. Agarose gel electrophoresis reagents and apparatus
13. 5× loading buffer
14. 10 U/µl T7 endonuclease I and 10× NEBuffer 2 (both from New England Biolabs)
15. DNA molecular-weight marker e.g. Gene Ruler DNA Ladder Mix (Thermo Scientific)
16. Bio-Rad Image Lab 4.1 (Bio-Rad) or ImageJ (National Institutes of Health, USA) software

Methods

The below-specified transduction experiments serve simply as an example to provide general guidelines for validating TALEN-encoding AdVs. Obviously, types and amounts of human target cells as well as vector genotypes and doses are at the discretion of the investigator.

1- One day prior transduction, seed 2.0×10^5 human myoblasts in wells of a 24-well plate and incubate the cells overnight at 37ºC in a 5% CO_2 atmosphere.

2- Co-transduce the target cells with 1:1 mixtures of $AdV^{\Delta2}$P.TALEN-$L^{S1}.F^{50}$ and $AdV^{\Delta2}$P.TALEN-$R^{S1}.F^{50}$. Preferably, to determine limiting doses and TALEN activity plateauing levels, apply a broad vector dose range onto the target cells. Negative controls can be provided by mock-transduced cells as well as by cells exposed at high doses to each AdV individually.

3- At 3 days post-infection, harvest the control and the co-transduced cell cultures and divide them in samples for protein expression and target site genotyping analysis. The assay for the former analyses has been detailed elsewhere (32), whereas that for the latter is as follows.

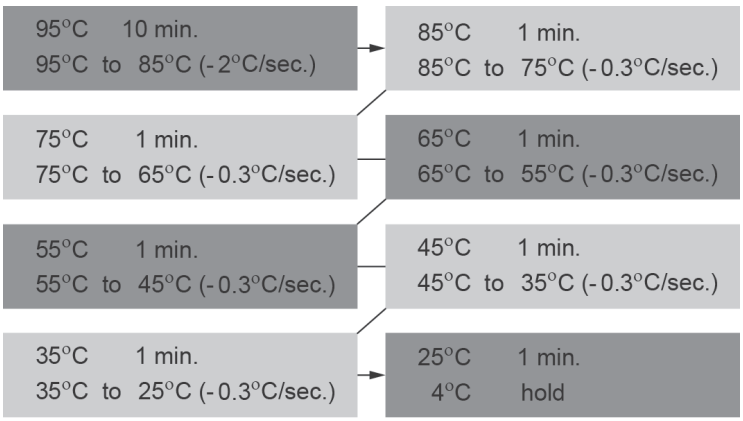
4- Initiate the total cellular DNA extraction procedure by incubating the target cells overnight at 55ºC in 500 µl of lysis buffer containing freshly added proteinase K at a final concentration

of 100 ng/ml. Next, subject the DNA to two rounds of phenol:chloroform:isoamylalcohol (25:24:1) extraction and to one round of chloroform extraction to remove traces of phenol. Next, add half volume of 7.5 M ammonium acetate and 2.5 volumes of ice-cold ethanol and precipitate the DNA by centrifugation at 20,000 ×*g* for 30 minutes at 4ºC. Wash the DNA pellets with 70% ethanol, after which air-dry and resuspend the DNA in 100 µl TE buffer pH 8.0 containing 100 µg/ml of DNase-free RNaseI.

6- Add 2 µl of the purified DNA to 50-µl PCR mixtures containing 0.4 µM of *AAVS1*-specific primers (5'-TTCGGGTCACCTCTCACTCC-3' and 5'-GGCTCCATCGTAAGCAAACC-3'), 0.1 mM of each dNTP, 1× Colorless GoTaq Flexi buffer, 1 mM $MgCl_2$ and 2.5 U GoTaq DNA polymerase.

7- Apply the following PCR cycling conditions: Initial denaturation for 5 minutes for 95ºC, followed by 40 cycles of 30 seconds at 95ºC, 30 seconds at 61ºC and 30 seconds at 72ºC. Terminate the reactions by an elongation period of 5 minutes at 72ºC.

8- Denature and re-anneal 20-µl amplicon samples by using a regular thermocycler machine and the following program:



9- Incubate the resulting DNA species in 25-µl reactions containing 5 U of T7 endonuclease I and 1× NEBuffer 2 for 20 minutes at 37ºC.

10- Stop the reactions by adding loading sample buffer containing SDS and immediately subject the DNA to electrophoresis through a 1.5% agarose gel in 1× Tris-Acetate-EDTA buffer.

11- Visualize by in-gel staining with a DNA dye (e.g. ethidium bromide) intact and digested amplicons resulting from NHEJ-mediated formation of small insertions and deletions (indels) at the TALEN target sequences.

12- Estimate the frequency of indel formation by densitometry of electrophoresis-resolved DNA molecules. To this end, commercially and publicly available software packages such as Bio-Rad Image Lab 4.1 and ImageJ, respectively, can be deployed.

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